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# Histological Effects and Prenatal Exposure to Crude Aqueous Extract of *Morinda lucida* Leaves on the Frontal Cortex of Growing Wistar Rats

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author OOA designed the study and wrote the protocol. Authors OOA, AAA and AAO managed the animals, collected all data, performed the statistical analysis and wrote the first draft of the manuscript. Authors OOA, AAA and AAO did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** The use of medicinal plants has always been part of human culture and is common in Africa. Amongst the medicinal plants commonly used in Nigeria for management/ treatment of various types of ailments is *Morinda lucida* Benth.
**Aims:** This research work was designed to investigate the effects of prenatal exposure of *Morinda lucida* on the frontal cortex in wistar rats.
**Methods and Materials:** A total of 25 pregnant wistar rats with an average weight of 150g were randomly divided into five groups (A-E) of five (5) rats each. Group E served as control and were given normal saline. Groups A, B, C and D were orally administered with *Morinda lucida* (400mg/kg) on the first, second, third and all weeks of pregnancy respectively. The litters in each group were

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then weighed and sacrificed by cervical dislocation on days 1, 7, 14, 21, 28 and 35 after birth. The brains were also weighed after sacrifice and the frontal cortex excised, fixed in formocalcium for routine histological techniques as well as homogenized in 0.25M sucrose solution, pH 7.4 for quantitative histochemical techniques. The photomicrographs of the frontal cortex of the control and the treated groups were observed and compared for changes and differences.

**Results:** The findings from this research work showed that for the brain stained with heamatoxylin and eosin the treated groups (A,B,C & D) were not different from the control group(E) in the development of their neurons as there were no alterations in their microarchitecture that is, no vacuolations suggesting cell death were seen and the cells appear well defined and also from the biochemical analysis, there are tissue damage which showed that there are no degradation, there are signs of normal development and there are proliferation and cell maturation.

**Conclusion:** From this study, it was found that *Morinda lucida* have no toxic or deleterious effect as it does not alter carbohydrate metabolism, does not cause any loss of Nissl substance and did not affect the microarchitecture of the neurons if administered during pregnancy.

*Keywords: Morinda lucida; Frontal cortex; teratogen; neurons; growing wistar rat; blood brain-barrier.*

## 1. INTRODUCTION

Many researchers in the field of embryology have in several years carried out researches in order to ascertain the teratogenicity of various chemical substances and herbs taken by women during pregnancy. This however has led to many of these substances/ herbs having potential of being teratogenic. *Morinda lucida* is among the widely used antimalarial plant around the world most especially in Africa and therefore its use among pregnant women cannot be undermined. Teratology is the branch of science that studies the abnormal development of embryo and the causes of congenital malformation [1]. It was believed until 1940s that the mammalian embryo developed in the impervious uterus of the mother but Gregg and Lenz made it apparent and acceptable that the developing embryo could be highly vulnerable to certain environmental agents that have negligible or non-toxic effects in adult individuals. Although the human embryo is well protected in the uterus by the embryonic/foetal membranes (amnion and chorion), and their mothers abdominal and uterine walls environmental agents may cause developmental disruptions following maternal exposure to them. These environmental agents are therefore referred to as teratogen. Awareness that certain agents can disrupt human prenatal development offers the opportunity to prevent some congenital anomalies; for example, if some are aware of the harmful effects of drugs (e.g. alcohol and some herbs), environmental chemicals, and some viruses, they will not expose their embryos to these teratogenic agents. The general objective of teratogenicity testing of drugs, chemicals, food additives and pesticides is to identify agents that may be teratogenic during human development and to alert physicians and pregnant women of their possible danger to the embryo/ fetus.

The use of medicinal plants has always been part of human culture and is common in Africa. In some countries, government encourages the use of indigenous forms of medicine rather than expensive imported drugs [2]. Also in Nigeria, a large percentage of the populace depends on herbal medicines because the commercially available orthodox medicines are becoming increasingly expensive and out of reach [3].

Amongst the medicinal plants commonly use in Nigeria for management/treatment of various types of ailments is *Morinda lucida*. It is a tropical West Africa rainforest commonly known as Brimstone tree [4]. *Morinda lucida* is a medium size tree that is about 15m tall with scaly grey bark, short crooked branches and shining foliage [5]. The leaves are used as beverages, which are usually taken orally for the traditional treatment of malaria, and as a general febrifuge, analgesic, laxative and antibiotic [6]. Two known triterpenic acids (Ursolic and oleanolic acids) were isolated from the leaves which are known to have protective effects on the brain. These also exhibit anti-microbial features against numerous strains of bacteria, HIV and HCV viruses and *plasmodium* protozoa causing malaria [7]. This research work aimed at investigating the effects of the leaf extract of *Morinda lucida* on the frontal cortex of growing wistar rats.

## 2. MATERIALS AND METHODS

### 2.1 Extract Preparation

The extraction was done by taken 50 g of the sample and soaking it directly in 400 mL of distill

water for 72 h. It was then filtered using cheese cloth and the solvent was removed using rotary evaporator and followed by drying on steam bath. The extract was then transferred into a sterile sample bottle and stored in a refrigerator until the time of administration.

**Preparation of the stock solution of the extract:** The stock solution was prepared just before use by dissolving 1 g of the aqueous extract in 10 mL physiological saline.

## 2.2 Experimental Design

A total of 25 females and 12 males adult wistar rats with average weight of 150g were used for this research work. The male rats were caged separately from the female rats. The females rats were randomly selected into five groups as follows A, B, C, D, and E each containing five rats. They were kept in the animal house of University of Ilorin, Nigeria and fed daily. The treatments for the various groups were administered accordingly. All experimental investigations were done in compliance with humane animal as stated in the "Guide to the care and use of Laboratory Animals Resources". National Research Council, DHHS, Pub.No NIH 86-23 [8] and in accordance with the guideline and approval of Nigeria Medical Ethical Association for Accreditation of Laboratory Animal Care.

## 2.3 Determination of Mating

Mating was done by natural copulation method. Vaginal smear test was performed on daily basis prior to mating. This was done in order to observe the oestrous cycle of the female rats which is on four phases – prooestrus, oestrus, dioestrus, and metoestrus. In rats, ovulation occurs in the oestrus phase. The vaginal smear was done by introducing a micro-pipette containing 0.5mL normal saline into the vaginal of the female rats. This was performed in the morning between 7.00am and 9.00am in order to get absolute result. The vaginal fluid was withdrawn with the pipette and placed on the microscope slide. This was viewed under the light microscope (Olympus UK Ltd, Essex, UK) to determine the presence of spermatozoa. A normal oestrus cycle takes a period of about 4-5 days [9]. At the proestrus and estrus phase, male rats were introduced into the female rats cages for mating to take place. This was done at a ratio 2:1 of females to males. The males were introduced at about 4:00pm and withdrawn at about 8:00am the next day. This was because copulation occurs more at night [9]. Marcondes' method of vaginal smear was carried out. Presence of vaginal plug or spermatozoa indicates mating and hence termed day 0 (zero) of pregnancy.

## 2.4 Animal Grouping

| | | | |
|---|---|---|---|
| A | 5 | Received normal saline | - |
| B | 5 | 0-7 days of pregnancy | 400mg/kg/bw |
| C | 5 | 8-14 days of pregnancy | 400mg/kg/bw |
| D | 5 | 15-21 days of pregnancy | 400mg/kg/bw |
| E | 5 | Receive extract throughout the pregnancy period i.e. 0-35 days | 400mg/kg/bw |

## 2.5 Animal Sacrificed and Sample Extraction

Twelve hours after the administration of the last *Morinda lucida,* the cervical dislocation was carried out following ethical humane animal euthanasia which was adopted with expertise cervical dislocation. The skulls were dissected by mid sagittal incision and the whole brain was excised. Regions of the frontal cortex was then excised out and processed for routine histological techniques sectioned at 3µm with a Rotary Microtome and stained using Heamatoxylin and eosin as described by Drury and Wallington, 1980 and Cresyl violet method as described by Venero et al. 2000.

## 2.6 Histological Preparation of Tissues

The brain tissues were fixed in 10% formol calcium. The tissues were processed and stained with Heamatoxylin and Eosin to demonstrate the microarchitecture of the cells, Cresyl fast violet to demonstrate Nissl substances as well as homogenized in 0.25M sucrose solution, pH 7.4 for quantitative for biochemical analysis to assess tissue damage in the brain. Photomicrographs were taken using a video digital camera (JVC, China) mounted on an Olympus light microscope (Olympus UK Ltd, Essex, UK) to demonstrate neurons and Nissl substance.

## 2.7 Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were analyzed using One-way analysis of variance [ANOVA]. A value of $p < 0.05$ was considered to be statistically significant.

## 3. PHOTOMICROGRAPHS DEMONSTRATION FOR H&E

Group A

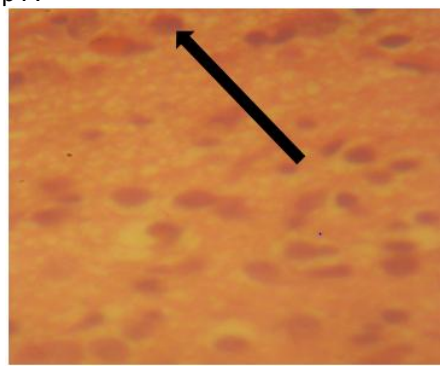

**Fig. 1. Histological demonstration of the frontal cortex using H&E staining techniques (×200) showing normal neurons (N, black arrow) at postnatal days 0-7**

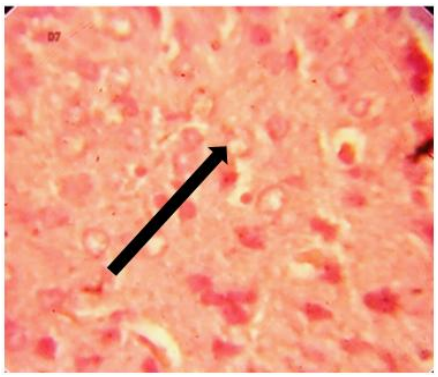

**Fig. 2. Histological demonstration of the frontal cortex using H&E staining techniques (×200) showing normal neurons (N, black arrow) at postnatal days 8-14**

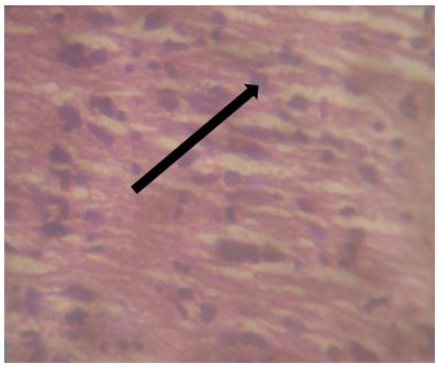

**Fig. 3. Histological demonstration of the frontal cortex using H&E staining techniques (×200) showing normal neurons (N, black arrow) at postnatal days 15-21**

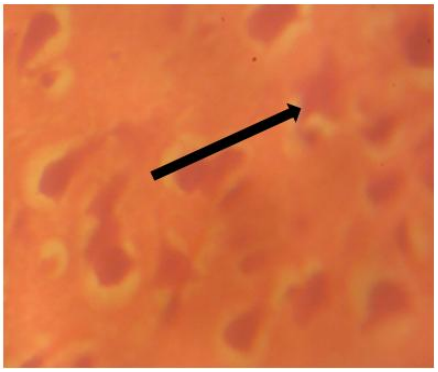

**Fig. 4. Histological demonstration of the frontal cortex using H&E staining techniques (×200) showing normal neurons (N, black arrow) at postnatal days 0-35**

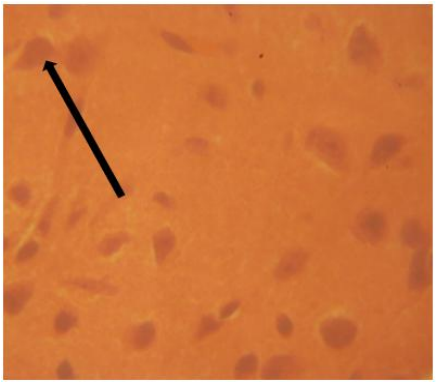

**Fig. 5. Histological demonstration of the frontal cortex using H&E staining techniques (×200) showing normal neurons (N, black arrow) showing control group**

### 3.1 Photomicrographs for Creysl Violet Stain

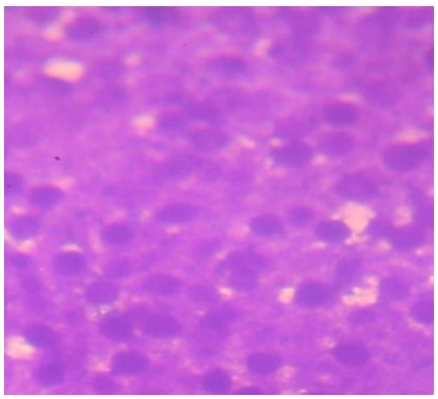

**Fig. 6. The extensive dark purple coloration indicating an abundance of Nissl bodies characteristic of a normal cell. Cresyl violet x200 at postnatal days 0-7**

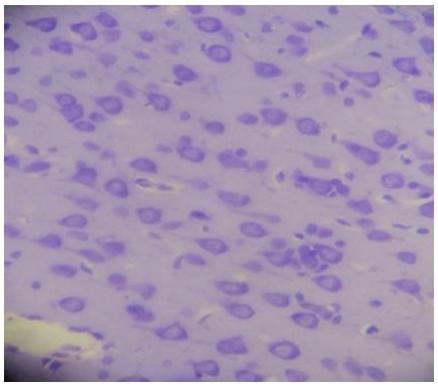

**Fig. 7. The extensive dark purple coloration indicating an abundance of Nissl bodies characteristic of a normal cell. Cresyl violet x200 at postnatal days 8-14**

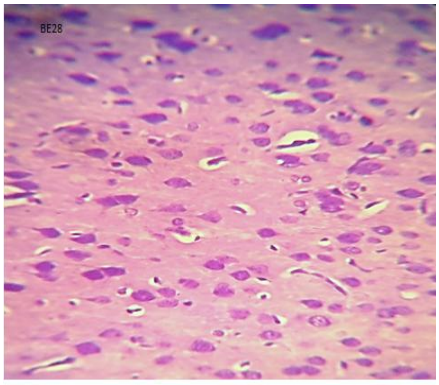

**Fig. 9. The extensive dark purple coloration indicating an abundance of Nissl bodies characteristic of a normal cell. Cresyl violet x200 at postnatal days 0-35**

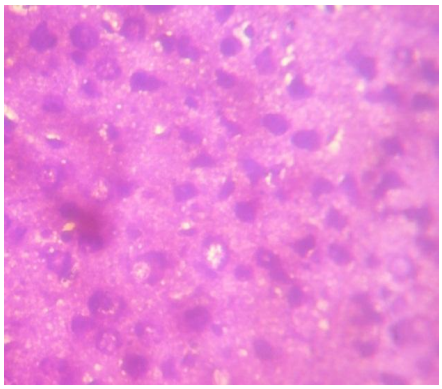

**Fig. 8. The extensive dark purple coloration indicating an abundance of Nissl bodies characteristic of a normal cell. Cresyl violet x200 at postnatal days 15-21**

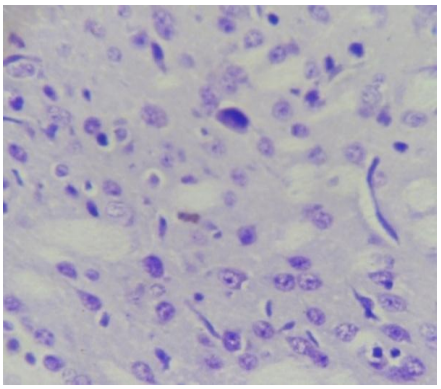

**Fig. 10. The extensive dark purple coloration indicating an abundance of Nissl bodies characteristic of a normal cell. Cresyl violet x200 at normal control group**

## 4. QUANTITATIVE HISTOCHEMICAL OBSERVATION

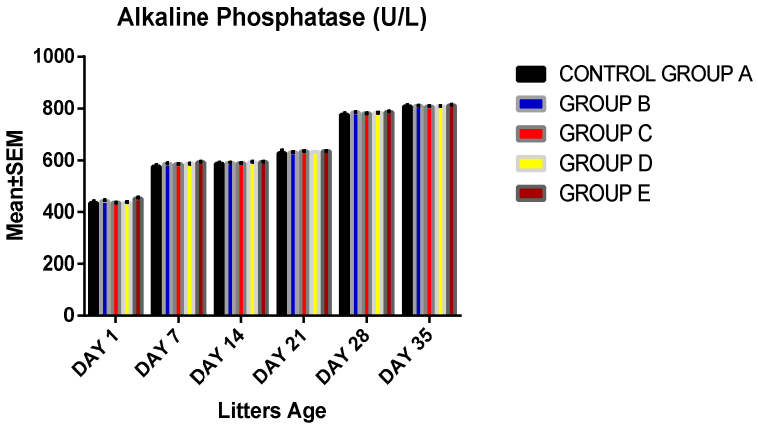


**Chart 1. Showing the level of alkaline phospatase (U/L)**
*Mean±SEM * p<0.05- Values For Alkaline phosphatase using ANOVA*

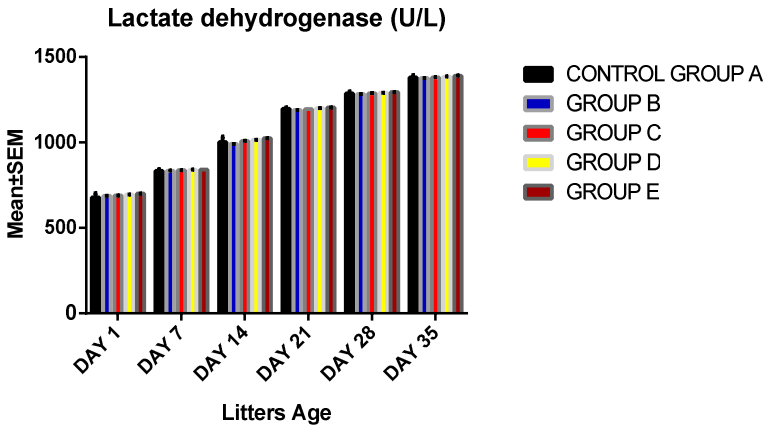


**Chart 2. Showing the level of lactate dehydrogenase (U/L)**

*Values are expressed as n=5 Mean±SEM *p<0.05- Values For Lactate Dehydrogenase using ANOVA*

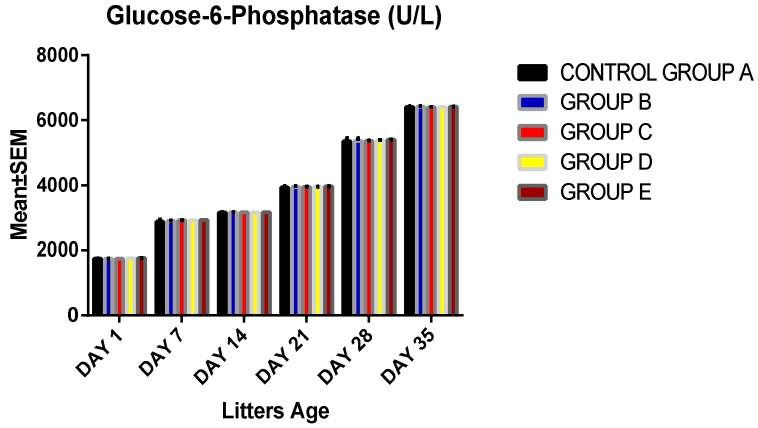


**Chart 3. Showing the level of glucose-6-phosphate dehydrogenase**

*Values are expressed as n=5 Mean±SEM, *p<0.05- Values For Glucose-6-Phosphate Dehydrogenase using ANOVA*

## 5. RESULTS AND DISCUSSION

Evidences seen in the photomicrographs are obtained from the histological part of this study because histology is good in early assessment of any brain damage caused by teratogenic agents. In the histological study, Heamatoxylin /Eosin were used to study the microarchitecture of the developing neurons while Cresyl fast violet was used to study Nissl substances.

Study with Heamatoxylin and eosin shows that the litters in the groups given *Morinda lucida* leaf extract were not different from the control group in the development of their neurons. As there were no alterations in their microarchitecture that is, no vacuolations suggesting cell death were seen and the cells appear well defined. This is supported by the low level of G-6-PDH in the litters whose mothers were given the *Morinda lucida* during pregnancy when compared with the control.

Nissl bodies manufacture and release certain chemicals, namely proteins [10]. The ultrastructure of Nissl bodies suggests they are primarily concerned with the synthesis of proteins for intercellular use [11]. The staining intensity of the Nissl substance in this study both in the control and in the treated group are similar which may suggest *Morinda lucida* did not affect the synthesis of protein for neuronal functions because there was no loss or degeneration of the Nissl substance– an indication that the extract administered to the mother rats during pregnancy has no neurotoxic effect on the frontal cortex of the litters.

The biochemical analysis done in this study are useful 'markers' for assessing brain damage as enzymes measurement are used to study and diagnose the presence of different diseases and abnormalities in the body [12].

Alkaline phosphatase (ALP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids and they are most effective in an alkaline environment [13]. ALP is resistant to inactivation denaturation and degradation, and also has a higher rate of activity it is believed to be a means for bacteria to generate free phosphate group for uptake and use is supported by the fact that ALP is usually produced by the bacteria during starvation and not when phosphate is plentiful [14].

There was no significant difference in the level of ALP in treated groups as compared to the control as seen in the chart which shows that there are no increases in the function of alkaline phosphatase. Therefore no degradation which is a major function of lysosomal enzymes and therefore no deleterious effect on the treated group which could have led to the death of the cells. Hence there is no need for the degradation of cell debris, or rather self- death (apoptosis) of cells and this is supported by the fact that alkaline phosphatase is usually produced by the bacteria only during phosphate starvation and not when phosphate is plentiful [14].

The levels of LDH in the treated group when compared with the control also shows that there are no irregularly rising and falling rates in metabolism, an indication of a normal development. This shows that there are no breakdowns or cell destruction in the brain as supported by Butt et al., 2002 that tissue breakdown elevates levels of LDH.

G6PDH is an enzyme in the pentose phosphate pathway. It converts glucose -6 –phosphate into 6- phosphoglucono-δ- lactone. It supplies reducing energy to cells by maintaining the level of co- enzyme nicotinamide adenine dinucleotide phosphate (NADPH). It is also known to function in glucose metabolism which is the primary source of energy needed to support life and this has been reported to increase in growing cells [15] and decrease in cell undergoing cell death [16].

The levels of G6PDH in the treated groups when compared with the control group shows no significant difference statically which suggest that there are no degradative enzymes but there are proliferation and cell maturation.

Quantitative histochemical analysis results correlated with the histological observations. The levels of alkaline phosphatase, lactate dehydrogenase and Glucose-6-Phosphate dehydrogenase were not higher in the treated group when statistically compared with the control. This is also shown in the histological result that it does not alter the microarchitecture of the neurons.

Since the above result show that *Morinda lucida* does not act as a teratogen to pups during pregnancy, it therefore suggests that the extract does not cross the placenta or the blood brain barrier to affect the developing embryo.

It is therefore concluded from the present study that aqueous extract of *Morinda lucida* have no toxic or deleterious effect, does not cause any loss of Nissl substance and did not affect the microarchitecture of the neurons if administered during pregnancy.

## 6. CONCLUSION

It can therefore be concluded from the present study that *Morinda lucida* does not cause any known effect on the frontal cortex of the growing wistar rats.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

All the authors hereby declare that all the experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in line with the ethical procedure laid down by Nigeria Medical Ethical Association for Accreditation of Laboratory Animal Care.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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